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FEBS Letters 585 (2011) 159–166

FEBS Letters

journal homepage: www.FEBSLetters.org

IL-8-induced neutrophil chemotaxis is mediated by Janus kinase 3 (JAK3)

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ARTICLE INFO

Article history:
Received 6 September 2010
Revised 15 November 2010
Accepted 16 November 2010
Available online 21 November 2010

Edited by Beat Imhof

Keywords:
Neutrophil
Inflammation
Proleukemic cell
Janus kinase 3
Kinase
Flavonoid

ABSTRACT

Janus kinase 3 (JAK3) is a non-receptor tyrosine kinase vital to the regulation of T-cells. We report that JAK3 is a mediator of interleukin-8 (IL-8) stimulation of a different class of hematopoietic relevant cells: human neutrophils. IL-8 induced a time- and concentration-dependent activation of JAK3 activity in neutrophils and differentiated HL-60 leukemic cells. JAK3 was more robustly activated by IL-8 than other kinases: p70S6K, mTOR, MAPK or PKC. JAK3 silencing severely inhibited IL-8-mediated chemotaxis. Thus, IL-8 stimulates chemotaxis through a mechanism mediated by JAK3. Further, JAK3 activity and chemotaxis were inhibited by the flavonoid apigenin (4′,5,7-trihydroxyflavone) at ~5 nM IC_{50}. These new findings lay the basis for understanding the molecular mechanism of cell migration as it relates to neutrophil-mediated chronic inflammatory processes.
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1. Introduction

The non-receptor tyrosine kinase Janus kinase 3 (JAK3) is vital to the regulation of T-cell signaling, lymphoid development and severe combined immunodeficiency (SCID) [1,2]. JAK3 is exclusively expressed in myeloid and lymphoid cell lines and in hematopoietic tissues like the thymus, spleen, bone marrow and fetal liver [3,4]. Mice lacking a catalytically intact JAK3 exhibit defects in B lymphocyte maturation and T lymphocyte activation [5,6]. Thymocytes and bone marrow progenitor cells from Jak $3^{-/-}$ mice have decreased chemotactic responses to the chemokines CXCL12 and CCL25 [7]. Additionally, leukemic cells require an active JAK3 enzyme in order to be killed by small molecule tyrosine kinase inhibitors [8]. Similar to other JAK family kinases, JAK3 contains seven JAK homology domains (JH) [9]. The JH1 domain is the putative kinase domain whose activity is regulated by tyrosine phosphorylation at Y980 and Y981. The pseudokinase JH2 domain is catalytically inactive and is rumored to interact with signal transducers and STAT proteins and negatively regulate the JH1 domain. The N-terminal JH6 and JH7 domains are implicated in binding to the gamma chain (γc) receptor and mutation at Y100 eliminates this interaction, which ultimately inhibits JAK3 activation. Additionally, JAK3 is implicated in signaling pathways of several cytokines that are involved in allergic airway disease/pulmonary inflammation (IL-2, -4, -7, -9 and -15) via phosphorylation of downstream STAT proteins [10–12], which directly links growth factor receptors to gene transcription.

Interleukin-8 (IL-8) is involved in several human diseases including inflammation, wound repair, angiogenesis, chronic obstructive pulmonary disease (COPD), atherosclerosis and cancer metastasis, and its primary target is induction of chemotaxis in granulocytic neutrophils and lymphocytes [13–18]. Besides inducing chemotaxis, IL-8 also induces changes in cytosolic calcium, neutrophil lipid metabolism, exocytosis and recruits neutrophils by binding and activating specific receptors, termed Cys-X-Cys-R (CXCR)-1 and -2 [19–23]. IL-8 mediated cell migration begins with polarization of neutrophils in the direction of the inflammation site followed by chemotaxis towards host- or pathogen-derived chemoattractants [24]. Neutrophils from stage I COPD patients have normal responses to IL-8, but in the more advanced stages of disease (II–IV), neutrophils showed markedly reduced spontaneous migration and chemotaxis in response to IL-8 [25].

Abbreviations: JAK3, Janus kinase 3; IL-8, interleukin-8; dHL-60, differentiated HL-60 cells; MBP, myelin basic protein

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doi:10.1016/j.febslet.2010.11.031

To date, there has been no analysis of JAK3 kinase activity of stimulated human polymorphonuclear neutrophils (PMN). Here, we have determined the effect of IL-8-mediated activation of JAK3 in human PMN and in the neutrophil-like differentiated HL-60 cells (dHL-60) and found that JAK3 is robustly involved in IL-8-iduced chemotaxis. Additionally, we are also demonstrating a potent effect of the flavonoid apigenin in neutrophil and HL-60 cell motility.

2. Materials and methods

2.1. Chemicals

Human IL-8 was from R&D Systems (Minneapolis, MN). Myelin basic protein (MBP) to be used as the MAPK substrate and S6 Kinase (RsK2) substrate peptide 2 (KKRNRTLTV) were from Millipore (Temecula, CA). The PKC substrate (QKRPSQRSKYL) and the JAK3 peptide substrate "Jak3tide" (GGEEEEYFELVKKKK) were from Upstate (Lake Placid, NY). Apigenin was from Sigma (St. Louis, MO).

2.2. Isolation of peripheral blood neutrophils and HL-60 differentiation (dHL-60)

Neutrophils were isolated from peripheral blood of human donors who had signed an IRB-approved consent form similar to Ref. [26] and were estimated to be >95% pure. HL-60 cells were maintained in Iscove's DMEM containing 40% fetal calf serum, 2 mM L-glutamine and penicillin/streptomycin. Cell density was maintained between 1 and 2×10^6 cells/ml. HL-60s were differentiated (dHL-60) for 4 days using 1.75% (v/v) DMSO in the complete growth media in order to achieve the expression of the neutrophilic phenotype. Both neutrophils and dHL-60 cells were ultimately each resuspended in HBSS at a concentration 1.5×10^6 cells/ml for use in chemotaxis assays or 1×10^7 cells/ml for use in both PLD and kinase assays.

2.3. dsRNA Transfection of dHL-60 cells

Twenty-four hours after induction of differentiation, HL-60 cells were transfected with 300 nM dsRNA's using nucleofection per the manufacturer's protocol (Amaxa, Gaithersburg, MD). Fresh DMSO to 1.75% (v/v) was added to the media post-nucleofection, and cells were cultured for an additional 72 h period. For JAK silencing, we used a "Selected validated" dsRNA from Applied Biosystems (Foster City, CA) that targeted exon 19; sense sequence: 5′-GUAUCGUGGUGUCAGCUAUtt-3′. For PKC silencing, we used a dsRNA from Santa Cruz Biotechnology (Santa Cruz, CA) that targeted five different exons specific for the PKC isoforms α, β, δ, μ and ι. The sequences target the following five regions: ACCAAGCAGAAGACCAACA; CACUGCACCGACUUCAUCU; UCAGUCCAUCAACAAGCAA; GGGAUGUGCAAAGAGAACA and CAGAGAAGCACGUGUUUGA. A negative control for all silencing was 100 nM siRNA "Neg-siRNA#2", purchased from Applied Biosystems. This control siRNA is a 19 bp scrambled sequence with 3′ dT overhangs (sequence not disclosed by Applied Biosystems) certified not to have significant homology to any known gene sequences from mouse, rat or human and causes no significant changes in gene expression of transfected cells after 48 h at the same concentration as the dsRNA in test.

2.4. Chemotaxis assays

Neutrophils or dHL-60s were resuspended at a concentration 1.5×10^6 cells/ml in chemotaxis buffer (HBSS + 0.5% bovine serum albumin). Two hundred microliters cells were applied to the upper chamber of 5 μm Transwells (24-well format) with a 6.5 mm diameter membrane. IL-8 (20 nM) in 500 μl of chemotaxis buffer was placed in the lower wells. Reactions were incubated for 1 h at 37 °C in a 5% CO_2 cell culture incubator. Results were quantified in triplicate as number of cells migrated per insert.

2.5. Kinase assays

Approximately 5×10^6 human neutrophils or dHL-60 cells were incubated with 20 nM IL-8 for 14 min at 37 °C. After stimulation, cells were sedimented, washed and finally lysed via sonication in 20 μl SLB containing protease inhibitors. Each lysate was incubated in the presence of the following final concentration of each: 8.25 mM HEPES, pH 7.5, 18.75 mM $MgCl_2$, 1.25 mM EGTA, 18.75 μM Na Orthovanadate, 3.125 μM *p*-nitrophenylphosphate (PNPP), 0.625 μCi [γ-^{32}P]-ATP, 40 μM cold ATP and the relevant kinase substrate to yield a 40 μl total kinase reaction volume. Cell lysates were immunoprecipitated with an antibody at 1 μg/μl intended to be used for the kinase assay (i.e., with anti-JAK3 for JAK3 kinase assay or with anti-PKC for a PKC kinase assay). For the specific isoforms of PKC, each sample was incubated with 10 μg of anti-myc-agarose, anti-PKCα-agarose, anti-PKCβ-agarose or anti-PKCδ-agarose at 4 °C for 2 h using a tube rotisserie. Each immunoprecipitate was washed and resuspended into 2×30 μl volumes of SLB and used in the kinase assays as listed above using no peptide substrate or the PKC peptide substrate only. To further account for specificity, each kinase reaction contained a relevant, specific kinase substrate. The concentration of those are as follows: no peptide negative control, 500 μM MBP as the MAPK substrate, 62.5 μM S6 Kinase substrate peptide-2, 10 μM active MAPK for mTOR, 62.5 μM PKC substrate and 42 μM JAK3tide substrate. All kinase reactions were incubated at 30 °C for 20 min. Reactions were stopped by spotting 20 μl reactions onto 2×2.5 cm^2 pieces of P81 Whatman filter paper for duplicate determinations. After washing all samples in filters were counted in a Beckman LS 6000TA liquid scintillation counter using the [^{32}P] protocol for 1 min each. Results were quantified as DPMs and expressed in terms of *-fold* activation.

2.6. Phosphorylation of H2B

Immunoprecipitations with anti-PKCα antibodies were conducted similar to [27]. After immunoprecipitation, kinase assay was performed by incubating anti-PKCα-agarose for 1 h at 37 °C in the presence of 20 μl kinase assay buffer (25 mM HEPES, pH 7.3, 10 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM cold ATP and 5 μCi [γ-^{32}P] ATP). To each reaction, 5 μg of histone H2B was added as exogenous substrate. Reactions were stopped by the addition of 10 μl 5× Laemmli buffer. Samples were boiled for 5 min and loaded onto a SDS–polyacrylamide gel.

2.7. Conditions for inhibition with apigenin

Approximately 5×10^6 human neutrophils or dHL-60 cells were incubated with the appropriate concentration of apigenin (in 1000-concentrated stock) for 20 min in a 37 °C water bath with vigorous shaking. After incubation with the inhibitor, cells were either mock-treated or treated with 20 nM IL-8 for 14 min at 37 °C. After stimulation, cells were sedimented and washed and lysed via sonication in 200 μl SLB containing protease inhibitors.

2.8. Statistical analysis

Data are presented as the mean + S.E. The difference between means was assessed by the single factor analysis of variance (ANOVA) test. Probability of $P < 0.05$ was considered to indicate a significant difference.

3. Results

3.1. IL-8 stimulates JAK3 activity of human PMN and leukemic cells

IL-8 stimulates the activity of JAK3 in human neutrophils in a time (Fig. 1A) and concentration dependent (Fig. 1B) fashion. Maximal phosphorylation of the JAK3 peptide substrate occurred at 5–7 min when using 20 nM IL-8 (Fig. 1A). Additionally, between 1 and 10 nM IL-8 provided optimal JAK3 phosphorylation at 7 min of time (Fig. 1B). The combination of immunoprecipitation with anti-JAK3 antibodies and the use of the JAK3 specific synthetic peptide substrate, GGEEEEYFELVKKKK, made it possible for us to ascertain that the isoform being conserved is indeed JAK3 (over JAK1 or JAK2). JAK3tide as an exclusive peptide substrate for JAK3 has been utilized by other authors [28], as it is phosphorylated by JAK3 at Tyr-7 and not by any other JAK family kinase.

We next compared the effect of IL-8 on JAK3 kinase activity between freshly isolated neutrophils and differentiated HL-60 that express the neutrophilic phenotype. We wanted to do this because subsequent experiments used molecular biology approaches (silencing with dsRNA and incubation for several days) that are not possible to evaluate with short-lived neutrophils. As indicated in Fig. 1C, IL-8 was able to stimulate the activity of JAK3 in neutrophilic dHL-60 albeit at a lower extent than that seen in fresh neutrophils, but still in a significant way over controls. When IL-8 was preincubated with antibodies to the IL-8 receptor (IL-8R) and subsequently utilized in the stimulation of cells, JAK3 phosphorylation was completely abrogated in both neutrophils and dHL-60s (Fig. 1C), indicating that the stimulation effect is mediated by the receptor of IL-8 specifically. Thus, we conclude that we are documenting a novel finding that JAK3 is present in neutrophils and can be specifically activated in vitro by IL-8 in both human neutrophils and differentiated leukemic dHL-60 cells.

3.2. IL-8-stimulated chemotaxis is mediated by JAK3

As known, IL-8 is a potent chemoattractant for neutrophils. Fig. 2A shows that it is also a chemoattractant for neutrophilic dHL-60 leukemic cells. The number of cells that migrated in response to 20 nM IL-8 increased in proportion to increasing time of migration (open triangles) compared to the non-stimulated cells (open circles). As a negative control, Fig. 2A also shows IL-8-

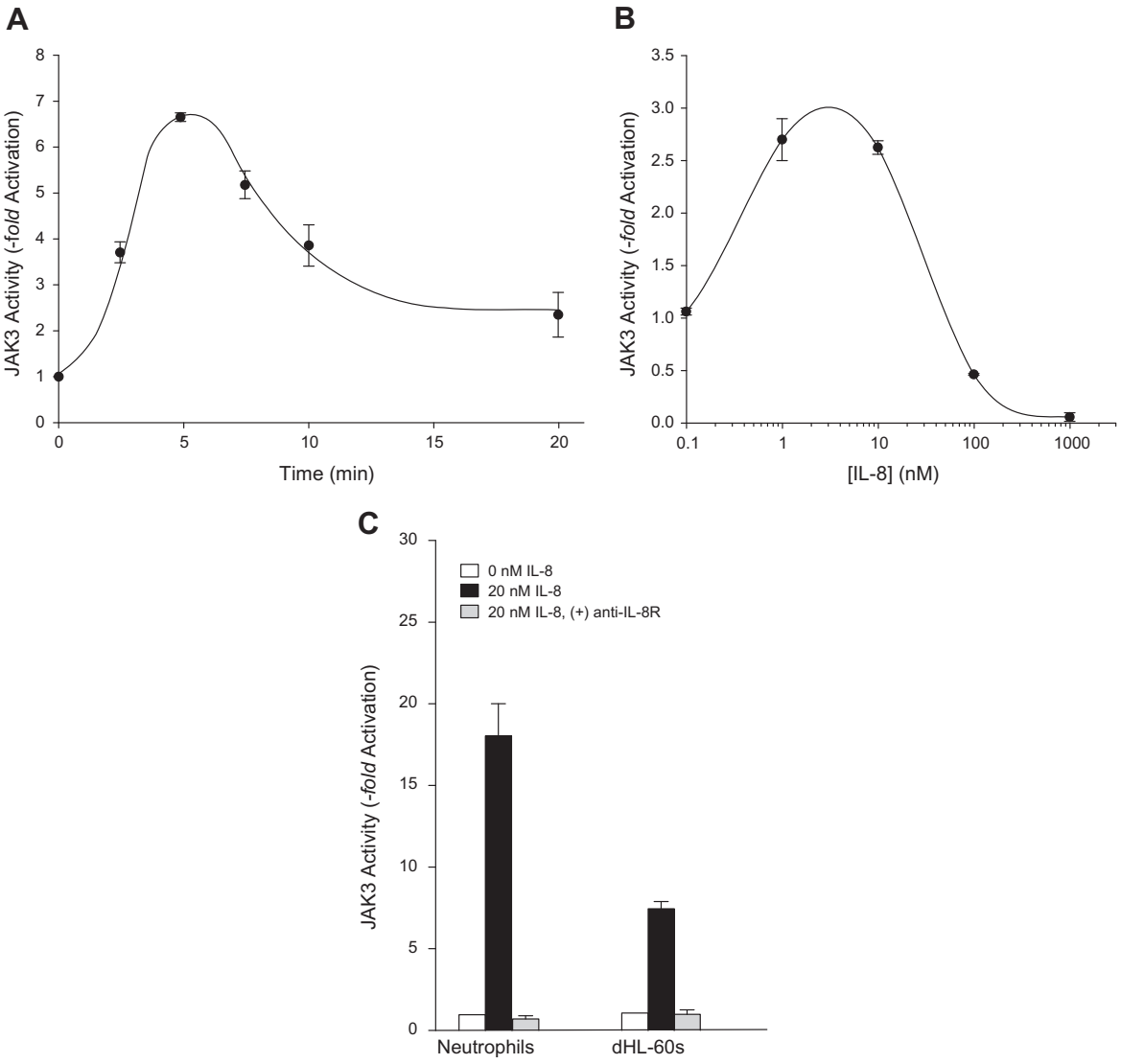

Fig. 1. IL-8 stimulates JAK3 activity of human neutrophils and differentiated HL-60 cells. Neutrophils were incubated without or with 20 nM IL-8, lysates prepared, immunoprecipitated with specific anti-JAK3 antibodies and JAK3 kinase reactions performed as in Section 2. (A) Time and concentration (B) response curves of JAK3 activity in neutrophils. (C) Effect of anti-IL-8R antibodies preincubated with 20 nM IL-8 prior to activation of JAK3 activity in neutrophils and dHL-60 cells.

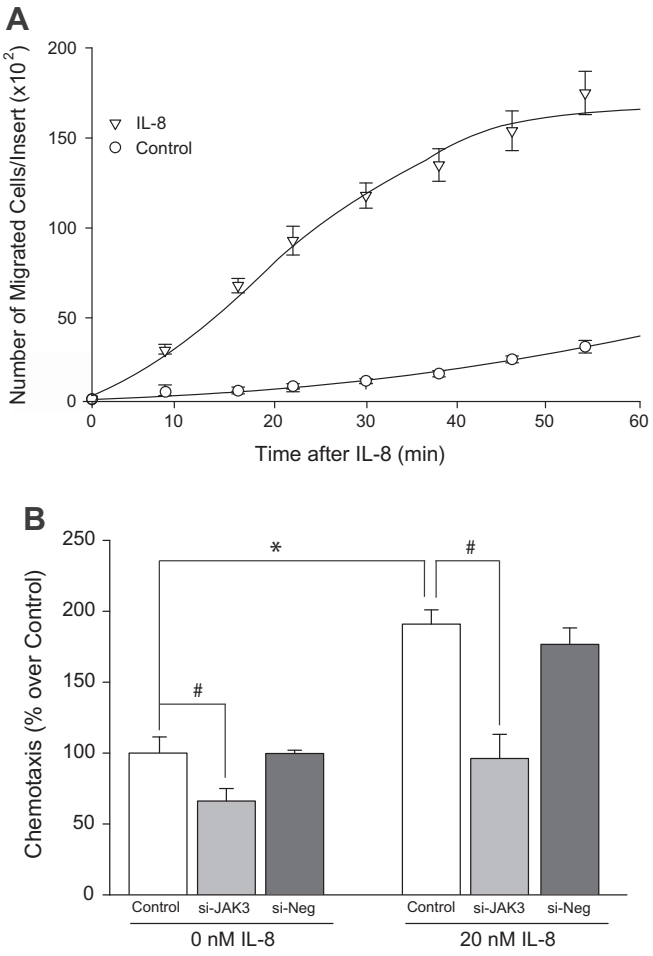

Fig. 2. IL-8-stimulated dHL-60 cell chemotaxis is mediated by JAK3. (A) Time course of IL-8-stimulated chemotaxis. Number of migrated cells/Transwell insert was measured as time elapsed from the addition of 20 nM IL-8 to the lower chambers of Transwell inserts. Results are the mean ± S.E. from three fields of view at 20× magnification. Control, untreated cells (open circles), cells treated with 20 nM IL-8 only (open triangles). (B) Effect of silencing JAK3 with dsRNA for 4 days on dHL-60 cell chemotaxis. Si-Neg is a scrambled dsRNA.

independent cell migration, chemokinesis, was detected in these cells but to a very limited and small extent (only about 20% of IL-8 stimulated cells at maximal time) (circles). Importantly, this effect was dependent on JAK3, as silencing with siRNA specific for JAK3 inhibited both basal chemokinesis by ~30% (Fig. 2B, left group of bars) and IL-8 activated chemotaxis by ~50% (Fig. 2B, right group of bars). This serves to indicate that IL-8-induced chemotaxis has a strong component that relies on JAK3 for intracellular signaling.

3.3. JAK3 activity is inhibited by apigenin

Next, we endeavored to further manipulate the IL-8-stimulated kinase activity with a small molecule inhibitor compound that could inhibit tyrosine kinases. We chose to use 4′,5,7-trihydroxyflavone (apigenin, Fig. 3A, inset) a plant polyphenol, flavonoid aglycone derived from green leafy vegetables [29–32]. Apigenin mediates inhibition of tyrosine kinase activities via interaction with the ATP-binding site of the specific kinase, which results in suppression of oncogene expression [27,33–40]. It also inhibits cell proliferation by arresting the cell cycle at G_2/M phase [41–44]. Using either neutrophils or dHL-60 cells, we show the positive effect of 20 nM IL-8 stimulation on JAK3 (Fig. 3A) activity in the

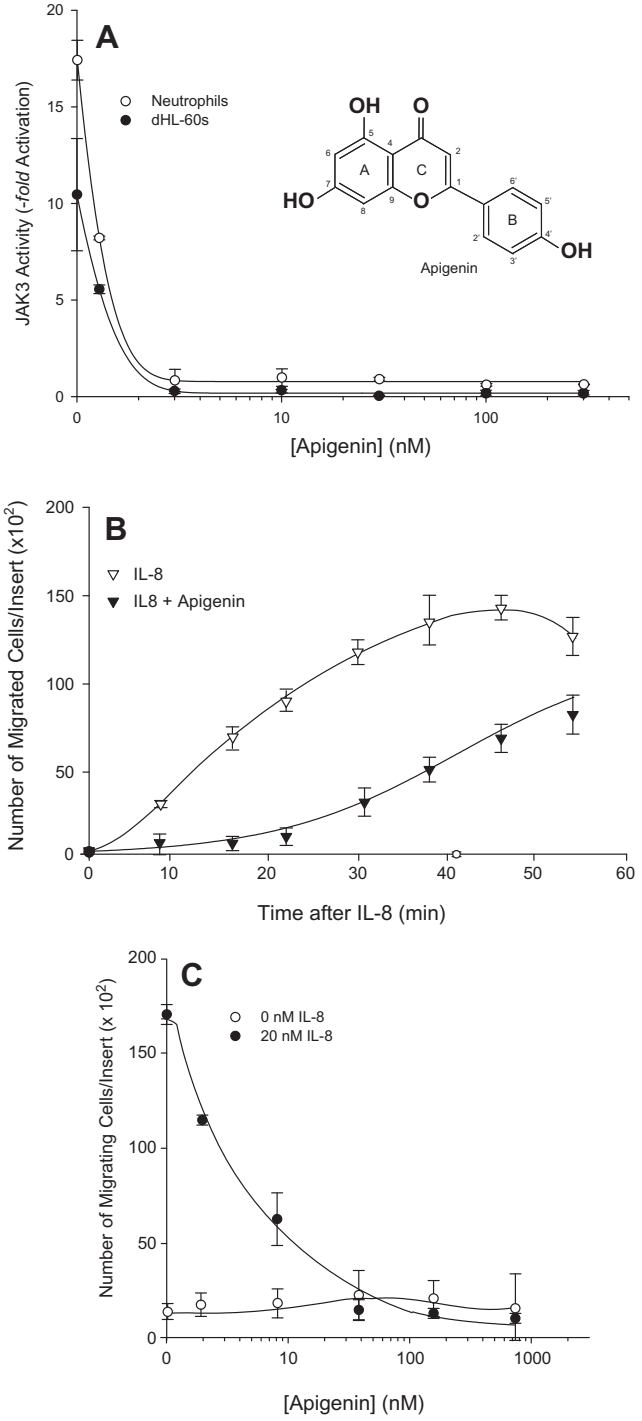

Fig. 3. Apigenin is a powerful inhibitor of both JAK3 activity and chemotaxis. (A insert) Schematic drawing of apigenin structure. Neutrophils or dHL-60 cells were treated with increasing apigenin for 20 min at 37 °C followed by 20 nM IL-8 stimulation for 10 min at 37 °C. (A) Effect of apigenin on JAK3 activity of neutrophils (open circles) or dHL-60 cells (filled circles). (B) Time course of IL-8-stimulated dHL-60 cell chemotaxis in the presence (filled triangles) or absence (open triangles) of apigenin. (C) Concentration dependent response of apigenin concentrations and effect on neutrophil chemotaxis. Unstimulated neutrophils (open circles), neutrophils stimulated with 20 nM IL-8 (filled circles).

absence of apigenin. Following incubation with increasing concentrations of apigenin, JAK3 phosphorylation activity drastically decreased to near baseline in both cell models. IC_{50} apigenin concentrations for JAK3 were 1.3 nM for neutrophils and 1.1 nM for dHL-60 cells. Although an IC_{50} concentration of apigenin for JAK3 has not been reported to date by any other group, the IC_{50}

concentration of apigenin for immunopurified phosphatidylinositol-3-kinase (PI3K) from human blood platelets has been determined to be much higher at 12 μM [36].

After measuring the effect of apigenin on JAK3 in vitro, we found that apigenin could inhibit IL-8-mediated dHL-60 cell chemotaxis in a time dependent manner, as evidenced in Fig. 3B. The number of cells that migrated in response to 20 nM IL-8 increased in proportion to increasing time of migration (open triangles). However, in the presence of 50 nM apigenin, IL-8-mediated cell migration was reduced by >70%, specifically at early times (10–30 min of chemotaxis) (filled triangles).

Next, we performed a concentration dependent experiment to ascertain the IC_{50} of apigenin in vitro during chemotaxis (Fig. 3C). Using increasing concentrations of apigenin up to 1 μM, we determined the IC_{50} concentration of apigenin to be 20 nM for human neutrophils cells (Fig. 3C) in response to <20 nM IL-8. The decrease in chemotaxis was drastic and significant. Additionally, the IC_{50} value for neutrophils was in the subnanomolar range

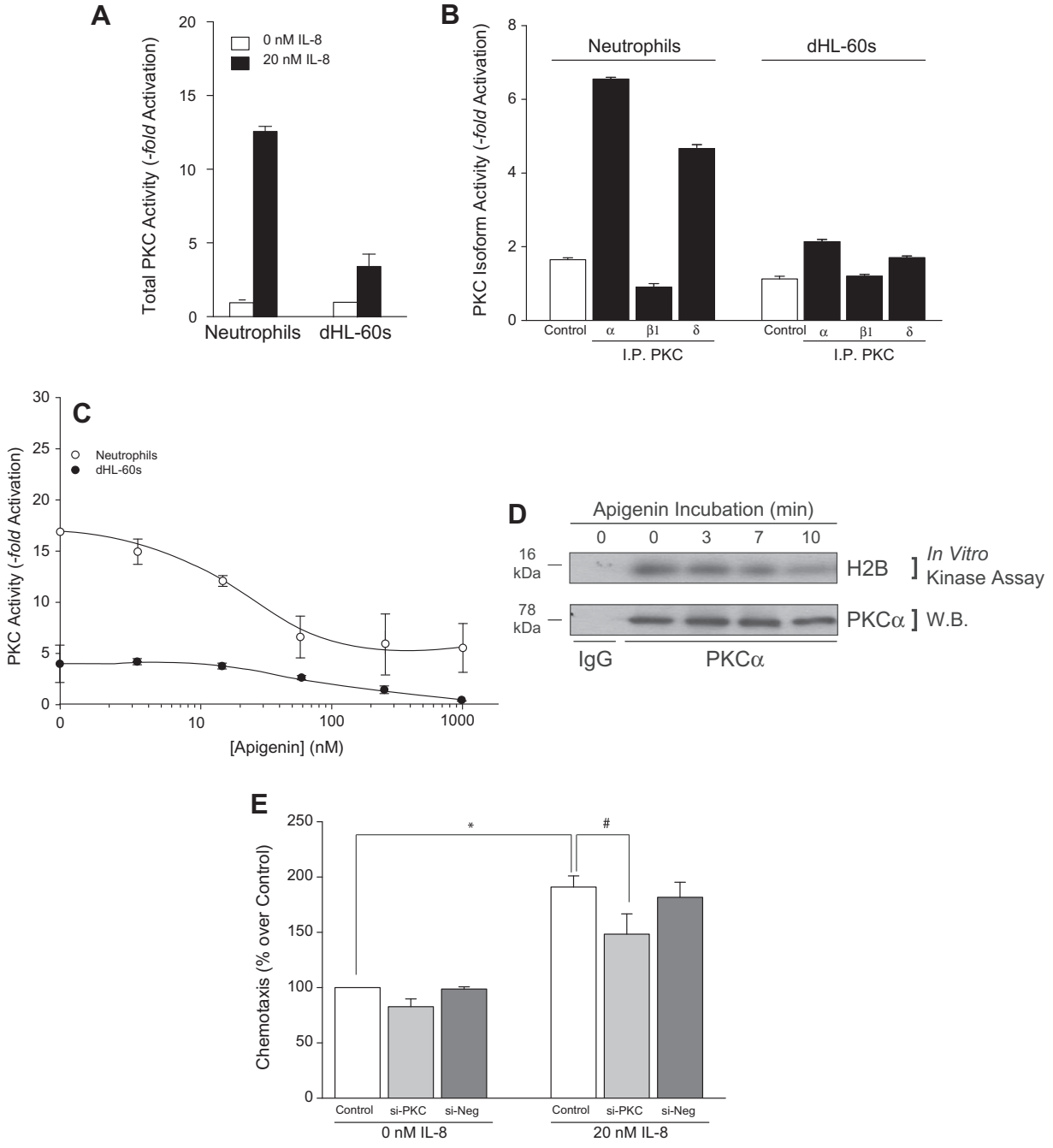

Fig. 4. IL-8 also activates PKC, albeit at a lower extent than it does JAK3. (A) IL-8 stimulates total PKC activity of human neutrophils and differentiated HL-60 cells. Cells were incubated without or with 20 nM IL-8, lysates prepared and immunoprecipitated and PKC kinase reactions performed as in Section 2. (B) Neutrophil and dHL-60 cell lysates were immunoprecipitated with anti-myc-agarose (negative control) or with α-PKCα-, α-PKCβ1- or α-PKCδ-agarose and used in the PKC kinase assay. (C) Effect of apigenin on PKC activity of neutrophils (open circles) and dHL-60 cells (filled circles). (D) dHL-60 cell lysates were immunoprecipitated with anti-PKCα antibodies or IgG control and subjected to in vitro kinase assay using H2B as substrate in the presence of [γ-^{32}P] ATP. Kinase reactions were resolved by SDS–PAGE, Western blot transferred and products visualized using autoradiography. Autoradiograph of increasing time of incubation with apigenin on PKCα phosphorylation of H2B (top panel). The same membrane was immunoblotted with anti-PKCα antibody to ensure equal protein loading control (bottom panel). (E) Effect of silencing PKC with dsRNA for 4 days on dHL-60 cell chemotaxis. Si-Control is a scrambled dsRNA.

indicating the extreme potency of apigenin to inhibit cell migratory functions. We also determined the IC_{50} concentration of apigenin to be 3.5 nM for dHL-60 cells in response to 20 nM IL-8 (data not shown). We report the effect of subnanomolar concentrations of apigenin on cell migration, while other groups have reported similar negative effects on cell migration assays but at ~1000-*fold* more apigenin (submicromolar range) [54,55]. These data suggest apigenin is a powerful inhibitor of chemoattractant-mediated cell migrations.

3.4. IL-8 stimulates other kinases: PKCα and PKCδ, albeit at a lower extent than JAK3

We have shown here for the first time that IL-8 stimulates JAK3 kinase activity in human neutrophils and the neutrophil-like human promyelocytic dHL-60 cells. Additionally, we wanted to determine how specific the effect of IL-8 stimulation was on JAK3. We performed similar kinase assays as those utilized for JAK3 (immunoprecipitation using specific kinase substrates as indicated in Section 2) and found that other kinases were activated by IL-8 in neutrophils; all but PKC exerted a lower extent of activation than JAK3. Further, we calculated the neutrophilic IC_{50} concentrations for apigenin inhibition for other kinases as follows: MAPK (1 μM), S6K (14 μM), mTOR (1 μM). All are 1–2 orders of magnitude greater than that of JAK3 as reported here.

Out of the four kinases, both the enhancing effect of IL-8 mediated by PKC and the inhibition by apigenin were somewhat close to that observed with JAK3, and we further explored PKC. Fig. 4A shows that the effect of IL-8 is in the range of what we have observed for JAK3 (Fig. 1C) albeit slightly lower. We next investigated which specific PKC subunits were involved in IL-8 signaling in human PMN and leukemic cells. Cell lysates were prepared and immunoprecipitated using antibodies specific for α-myc (a negative control reaction) and three different subunits of PKC: PKCα, PKCβ1 and PKCδ. As indicated in Fig. 4B, PKCα and PKCδ were detected in both IL-8-stimulated neutrophils and dHL-60 cells. PKCδ and PKCα were about 3.5-*fold* greater in neutrophils and 1.5-*fold* greater in dHL-60 cells when each was compared to their respective negative controls, respectively. Peak activity of each kinase was achieved at 10–14 min of incubation time (data not shown). In general, PKCα and PKCδ activities were more robustly measured in human neutrophils when compared to dHL-60 cells. PKCβ1 kinase activity was equal to or fell below that of the negative control α-myc immunoprecipitates and, therefore, was not readily quantifiable in either cell line using our experimental design.

PKC phosphorylation activity decreased as well in the presence of apigenin but not to the same extent as JAK3 activity (Fig. 4C). IC_{50} apigenin concentrations for total PKC activity were 15 nM for neutrophils (open circles) and 10-*fold* greater (200 nM) for dHL-60 cells (filled circles). Previously, it has been reported by Huang et al. that the IC_{50} concentration of apigenin for PKC is 10 μM in murine embryonic fibroblasts [37]. The data presented here indicate that neutrophils are more sensitive to apigenin than fibroblasts [36,37]. As PKCα was the PKC isoform that was activated to the greatest extent in both neutrophils and dHL-60 cells, we chose to determine the level of PKCα activity of an actual downstream protein substrate and not a synthetic peptide substrate (histone-2B, H2B). As shown in Fig. 4D, PKCα was able to phosphorylate the protein substrate H2B when stimulated with IL-8. When in the presence of 50 nM apigenin, markedly less phosphorylation of H2B by PKCα was evident. After 10 min of incubation with apigenin, H2B phosphorylation by PKCα decreased by more than 50% when compared to the PKCα loading control.

Lastly, the PKC-mediated component of PKC activation does not seem to play as important a role in chemotaxis as does JAK3, as silencing with dsRNA specific for PKC had a marginal effect on chemotaxis (Fig. 4E). Silencing with siRNA specific for PKC inhibited basal chemokinesis by only ~15% (Fig. 4E, left group of bars) and IL-8 activated chemotaxis by ~20% (Fig. 4E, right group of bars).

4. Discussion

The non-receptor tyrosine kinase JAK3 is vital to the regulation of T-cell signaling, lymphoid development and severe combined immunodeficiency (SCID), which contributes to it being considered a potential and worthwhile pharmacological target. Recently, JAK3 has specifically been implicated in myeloid cell development, as mutations to JAK3 have been found in acute megakaroblastic leukemia [45]. Thymocytes and bone marrow progenitor cells from Jak $3^{-/-}$ mice have decreased chemotactic responses to the chemokines CXCL12 and CCL25 [7]. The N-terminal FERM and possibly the SH2-like domains of JAK proteins regulate catalytic activity and/or are involved in binding to and association with relevant cytokine receptors and are considered vital to the initial steps involved in cytokine-mediated signaling [9,46,47]. The conversion of the extracellular binding signal following binding to the receptor of interest into a cascade of tyrosine kinase activity is mediated predominantly by JAK proteins. JAK3 is specifically implicated in signaling pathways of several interleukin cytokines that are involved in allergic airway disease/pulmonary inflammation via phosphorylation of downstream STAT proteins [10–12].

It has been shown that airway epithelial cells secrete various chemokines, cytokines, extracellular matrix proteins and lipid mediators, one of which is IL-8 that is already known to attract neutrophils and other hematopoietic cells to the site of inflammation [48–50]. IL-8 is involved in several human diseases including inflammation, and its primary target is induction of chemotaxis in granulocytic neutrophils and leukocytes, which are appropriate cell motility models for inflammation, tumor migration or wound healing. JAK3 has been found to have a pivotal role in mast cell-mediated bacterial clearance and neutrophil recruitment via regulation of TNF from mast cells [51].

Unlike G-CSF which cannot activate JAK3 in human neutrophils [52], we have shown for the first time that JAK3 can be correlated to human neutrophilic function in a time and concentration dependent manner and via the IL-8 signal transduction pathway, which implicates a role for JAK3 in IL-8-mediated inflammatory processes and cancer. Upon further determination, both PKCα and PKCδ were found to be IL-8-activated to a greater extent in neutrophilic lysates than in leukemic cell lysates following immunoprecipitation with antibodies specific to either kinase. JAK3 is more robustly activated in neutrophils by IL-8 than other kinases like PKC, MAPK, mTOR or p70S6K, which further implicates a JAK3 mechanism in IL-8-stimulated chemotaxis.

We have shown here JAK3 and PKCα phosphorylation activities in both neutrophils and leukemic cells following IL-8 stimulation are negatively affected by apigenin action. Additionally, we have associated the presence of nanomolar concentrations of apigenin to negative effects on random and chemoattractant-stimulated cell migration of neutrophils and leukocytes in a time and concentration dependent manner. It has been determined that submicromolar concentrations of apigenin suppressed by a factor of approximately 9-*fold* the release or production of proinflammatory cytokines, including IL-8, in human monocytes [53]. Apigenin has been found to severely hinder cell migratory processes, particularly adhesion, chemotaxis, invasion and metastasis in ovarian, breast, melanoma lung, cervical and prostate cancers [54–59]. Conventional wisdom suggests that apigenin is a pleiotropic effector that affects protease-dependent invasiveness and associated processes, proliferation of tumor cells and actin cytoskeleton organization, which exerts an anti-tumorigenic effect in vivo via inhibition of tumor cell penetration into healthy tissue.

In conclusion, our results provide documentation of the novel effect of IL-8-stimulated JAK3 activation in human neutrophils and leukemia. We have documented a previously defined molecular mechanism relevant to inflammation and cancer that also uniquely regulates JAK3. We have demonstrated that apigenin interferes with IL-8-mediated chemotaxis of human neutrophils and leukocytes that can be correlated to suppression of certain tyrosine kinases, namely JAK3, PKCα and PKCδ.

Additionally, we have been able to correlate a role for JAK3 in leukemic cell migration as transfection of JAK3 siRNA into dHL-60 cells severely inhibited chemotactic cells in response to IL-8. As apigenin effectively binds to JAK3 and suppresses this kinase moiety, information presented here could be used to design new chemotherapeutic strategies against IL-8-mediated inflammation and leukemia, potentially including use of the IL-8 receptor.

Acknowledgements

We thank Dr. Larry Ream for providing technical assistance. The grant HL056653 (J.G.-C.) from the National Institutes of Health has supported this work and NHLBI-HL 075040 (A.I.D.).

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